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Research paper

Avian follicular and interdigitating dendritic cells: Isolation and morphologic, phenotypic, and functional analyses☆

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ABSTRACT

An antiserum against *Eimeria tenella* sporozoites was used to localize and isolate Ag-binding cells in intestinal cecal tonsils of parasite-infected chickens. Based on their tissue localization, ultrastructural features, and expression of surface markers, two subpopulations of cells were isolated, $CD45^+$ interdigitating dendritic cells (IDCs) and $CD45^-$ follicular dendritic cells (FDCs). IDCs expressed MHC class I, MHC class II, and selectin, induced the proliferation of allogeneic naïve $CD4^+$ T cells, and increased the secretion of IFN-γ by autologous T cells. FDCs expressed surface IgG, IgM, ICAM-1, and VCAM-1, stimulated the proliferation of LPS-treated allogeneic B cells, and augmented the secretion of IgG by LPS-treated autologous B cells. Final cell yields were 6×10^5 to 8×10^5 cells per chicken with >95% purity. In summary, this combination of methods using Abs against *E. tenella* and CD45 made it possible for the first time to obtain a highly enriched IDCs and FDCs which are functionally active in chickens. This novel method will enable the detailed biochemical and immunological characterizations of avian dendritic cells and facilitate the investigation of their role in initiating immune response in normal and disease states.

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1. Introduction

Dendritic cells (DCs) are highly efficient APCs found in tissues that are in contact with the external environment, as well as in the bone marrow. Once activated, DCs migrate to the lymphoid tissues where they interact with T and B lymphocytes as APCs to initiate primary immune responses. Avian DCs were first identified in the cecal tonsils, secondary lymphoid organs located in the intestinal mucosa and containing T cell-dependent diffuse lymphoid regions and B cell-dependent germinal centers (Olah and Glick, 1979; Befus et al., 1980; Hoshi and Mori, 1973). In addition to macrophages and B cells, two additional, phenotypically distinct populations of APCs have been identified in avian lymphoid tissues, interdigitating DCs (IDCs) and follicular DCs (FDCs) which are found in the T- and B-dependent areas and participate in Ag presentation and activation of the respective lymphocytes (Gallego et al., 1995a; Del Cacho et al., 1995, 2008; Glick and Olah, 1984; Eikelenboom et al., 1983; Jeurissen, 1993; Gallego et al., 1992). In the gut, activation of intestinal DCs is required for the efficient generation of a host protective immune response to microbial infections (Del Cacho et al., 1993b). Despite the important roles that FDCs and IDCs play in the immune response, there is a lack of phenotypic and functional studies compared with

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Abbreviations: IDCs, interdigitating dendritic cells; FDCs, follicular dendritic cells.

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mammalian systems due to the absence of isolation protocols for avian DCs.

The apicomplexan protozoan *Eimeria* is the etiologic agent of avian coccidiosis, a major parasitic disease of poultry that is indigenous to most regions of the world. Coccidia parasites invade the intestinal mucosa and cause epithelial cell damage leading to diarrhea, poor feed conversion, and reduced body weight gain. While conventional disease control methods have relied on chemoprophylaxis with anti-coccidia drugs, novel methods are sought due to increasing governmental restrictions on the commercial use of coccidiostats, the appearance of drug resistant parasites, and the high costs of new drug development. Vaccination with live and attenuated parasites offers an alternative approach to disease control, but lack of cross-protection among the seven *Eimeria* species that cause coccidiosis, and the emergence of novel antigenic field variants in vaccinated flocks, are problems that are yet to be solved. Our approach to generating an improved coccidiosis vaccine has been to identify and characterize host immune pathways conferring broad-scale protective immunity, from initial pathogen exposure and Ag uptake/presentation to subsequent effecter mechanisms of cellular and humoral immunities. As part of this systematic approach, in the present study we isolated and characterized gut DCs following experimental *Eimeria* infection. To obtain high numbers of the intestinal DCs, chickens were orally infected with *Eimeria tenella* sporozoites based on our previous reports which showed enhanced numbers of DCs in the cecal tonsils compared with other lymphoid organs (Del Cacho et al., 2008). Sporozoite Ag-binding cells in the infected cecal tonsils were detected *in vitro* using a polyclonal antiserum against *E. tenella* Ags, the cells were isolated by FACS using the same antiserum, separated into $CD45^+$ and $CD45^-$ subpopulations, and characterized according to morphologic, phenotypic, and functional parameters. Based on these analyses and the known properties of avian DCs as described in the literature, we propose that the two cell populations represent IDCs and FDCs, respectively.

2. Materials and methods

2.1. Abs

Abs to chicken CD3 (clone CT-3), CD4 (clone CT-4), CD5 (clone 2-191), CD8 (clone CT-8), CD28 (clone AV7), CD44 (clone AV6), CD45 (clone LT40), Bu-1 (clone 21-1A4), IgG (clone G-1), IgM (clone M-4), IgA (clone A-1), MHC class I (clone F21-2), MHC class II (clone 2G11), and monocytes/macrophages (clone KUL01) were from Southern Biotech (Birmingham, AL). Ab to chicken CD41/CD61 (clone 11C3) was from Serotec (Kidlington, UK). Ab to chicken CD51/61(clone LM609) was from Chemicon (Hampshire, UK). Abs to human CD79 (clone HM57) and CD156 were from AbD Serotec (Oxford, UK). Ab to human VCAM-1 (clone BBIG-V1) and ICAM-1 were from R&D Systems (Minneapolis, MN). The Abs to the human proteins are cross-reactive with the homologous chicken proteins, against which no Abs are commercially available. Non-immune isotype-matched control Ig, and Alexa Fluor 488 and Alexa Fluor 647-labeled anti-chicken IgG and anti-human IgG Abs were from Invitrogen (Paisley, UK). Fluorescein-conjugated goat anti-rabbit IgG Ab was from Dako Laboratories (Glostrup, Denmark).

2.2. Parasites and experimental infections

Oocysts of an *E. tenella* strain originally obtained from Merck Sharp and Dome (Madrid, Spain) were propagated, isolated, and sporulated using standard procedures (Raether et al., 1995). One-day-old White Leghorn chickens were obtained from a commercial hatchery (Doux Iberica, Zaragoza, Spain) and reared in a coccidia-free environment until use. Four-week old chickens ($N = 50$) were infected with freshly prepared sporulated oocysts (stored for less than 4 weeks) by oral inoculation into the crop. Group 1 chickens were given a single dose of 5000 sporulated oocysts whereas group 2 served as non-infected controls. At 7 days post-infection, the animals were sacrificed by cervical dislocation and the cecal tonsils were removed. All experiments were reviewed and approved by the Animal Ethics Committee of the University of Zaragoza.

2.3. Preparation of E. tenella antiserum

Excysted sporozoites were separated from oocysts and sporocyst debris and sonicated on ice. The sonicate was centrifugated at $300 \times g$ for 5 min and the pellet was resuspended in 25 mM Tris–HCl, 150 mM NaCl, 5.0 mM EDTA, 1.0% Triton X-100, and protease inhibitor cocktail at 4 °C. The sample was centrifuged at $100{,}000 \times g$ for 1 h at 4 °C and the pellet was resuspended in the same buffer for 1 h at 4 °C and recentrifuged. Pellets were collected, protein concentrations were determined according to the procedure of Lowry et al. (1951) using BSA as the standard, and stored at −20 °C. Rabbits were subcutaneously immunized 5 times at 2-week intervals with 150–200 μg of *E. tenella* Ag emulsified with 0.25 ml of CFA. Serum samples were collected 12 days after each injection and the presence of parasite-reactive Abs was confirmed by ELISA.

2.4. Western blot analysis

Sporozoites were sonicated, centrifuged at $100{,}000 \times g$ for 20 min, the pellets were dissolved in reducing SDS-PAGE sample buffer and proteins were resolved on a discontinuous gel system using standard protocols (Laemmli, 1970). Proteins were electrophoretically transfered to nitrocellulose at room temperature for 18 h at 40 mV. The membrane was blocked in PBS containing 4% non-fat dry milk, incubated with the *E. tenella* antiserum (1:100) for 1 h at room temperature and peroxidase-conjugated goat anti-rabbit IgG Ab, and developed with diaminobenzidine.

2.5. Immunohistochemistry

Ethanol-fixed sporozoites were incubated with 1.7% H_2O_2 in 100% ethanol for 30 min to inactivate the endogenous peroxidase. The sporozoites were washed with PBS, pH 7.2, blocked with normal horse serum for 10 min, followed by incubation with *E. tenella* antiserum (1:100) for 90 min. Sporozoites were washed 3 times with

PBS and incubated with biotinylated anti-rabbit IgG Ab (Vector, Burlingame, CA) for 30 min. An avidin–peroxidase complex was applied for 45 min and the peroxidase reaction was developed with diaminobenzidine as described by the manufacturer (Vector). Sporozoites were incubated with normal rabbit serum as a negative control.

2.6. Immunofluorescence

Cecal tonsils were frozen in liquid nitrogen, cryostat sections were made and blocked with normal horse serum for 10 min, followed by incubation with *E. tenella* antiserum (1:100) for 18 h at 4 °C and fluorescein-conjugated goat anti-rabbit IgG Ab for 30 min. Fluorescence was analyzed with a BH-2 fluorescence microscope (Olympus, Hamburg, Germany). Two negative controls were used, cecal tonsil sections from uninfected chickens and sections from infected chickens stained with normal rabbit serum.

2.7. Transmission electron microscopy

Cecal tonsils were fixed with 0.05% glutaraldehyde in Bouin solution for 60 min at room temperature and embedded using the Durcopan water soluble kit according to the manufacturer's instructions (Bio-Rad, Hemel Hempstead, UK). Tissue sections (40–60 nm) were made using an ultramicrotome (LKB, Bromma, Sweden), the sections were stained for the presence of *E. tenella* parasites by immunohistochemistry as described above, subsequently stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy as described (Del Cacho et al., 2008). Negative controls were as described above.

2.8. Isolation of E. tenella Ag-binding cecal tonsil cells

Cecal tonsils were homogenized for 1 h at 37 °C in GKN (PBS supplemented with 11 mM D-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM Na_2HPO_4, 5.5 mM NaH_2PO_4) (Stumbles et al., 1998) supplemented with 0.2% BSA, 1.0 mg/ml collagenase A (Roche, Mannheim, Germany), and 750 U/ml bovine pancreatic DNase I (type IV, Sigma, St. Louis, MO) with continuous agitation. The released cells were filtered through a 250 μm mesh screen, resuspended and washed in GKN solution containing 5.0 mM EDTA, and passed through a 70 μm cell strainer (BD Falcon, Franklin Lakes, NJ). The cells were purified from dead cells, erythrocytes, and epithelial cells by Percoll density gradient centrifugation (1.075 g/ml, high density, Amersham Pharmacia Biotech, Uppsala, Sweden) at 950 g for 20 min. Cell viability was >90% by trypan blue exclusion. Isolated cells (1.0×10^7) were sequentially incubated on ice for 30 min with 500 μl of *E. tenella* antiserum (1:100) and fluorescein-conjugated goat anti-rabbit IgG Ab. Cell sorting was performed using an Epics Elite flow cytometer (Beckman Coulter, Fullerton, CA) equipped with an argon ion laser set at 488 nm with 20 mW and fluorescence was detected with a band pass filter (525 ± 15 nm). Data files were analyzed with software provided with the flow cytometer (version 4.02). Each experiment included positive and negative controls (cells incubated with normal rabbit IgG or without primary Ab) to discriminate the labeled cell population from the background debris. Sorted cells were examined by phase contrast microscopy (Nikon, Tokyo, Japan).

2.9. Magnetic separation of CD45$^+$ and CD45$^-$ cells

E. tenella Ag-binding cecal tonsil cells isolated by FACS were incubated with biotinylated anti-chicken CD45 Ab (1:100) for 45 min, washed, and isolated using Dynabeads FlowComp according to manufacturer's guidelines (Invitrogen). Post-separation cell viability was >90%. CD45$^+$ and CD45$^-$ cells were cultured in RPMI 1640 (Sigma) supplemented with 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, 40 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated chicken serum (Sigma) for cell proliferation and IFN-γ and IgG secretion assays.

2.10. Phenotypic analyses

Isolated CD45$^+$ or CD45$^-$ cells (5.0×10^4) were pre-incubated with normal horse serum (Vector) for 10 min, followed by incubation for 90 min with 0.2 ml of primary Ab or non-immune isotype-matched control Ig (1:100). The cells were washed, incubated for 30 min with Alexa Fluor 647-rabbit anti-mouse IgG Ab (1:100), and analyzed by FACS as above with an argon ion laser set at 595 nm and fluorescence determined at 650 ± 15 nm. A total of 20,000 live gated cells, on the basis of forward- and side-light scatter, were analyzed for each sample.

2.11. Isolation of macrophages, T cells, and B cells

Macrophages were elicited by intraperitoneal injection of naïve chickens with 10 ml of 3% (w/v) Sephadex G-50 (Sabet et al., 1977), the cells were collected by intraperitoneal lavage with 40 ml of sterile HBSS, and isolated by centrifugation on lymphocyte separation medium (ICN Biomedicals, Solon, OH) at 250 g for 20 min at room temperature. The cells were washed with Ca^{2+}- and Mg^{2+}-free HBSS and macrophages were isolated by plastic adherence. PBMCs (2×10^7) from uninfected animals were incubated with pre-titrated quantities of anti-CD4 or anti-IgG mAbs (100 μl) on ice for 45 min, washed with HBSS containing 3% BSA, and incubated with biotinylated goat anti-mouse IgG Ab (1.0 μg/10^6 cells) (BD IMag Cell Separation System, BD Biosciences, San Jose, CA) for 15 min on ice. After washing, streptavidin-labeled magnetic particles (40 μl/ml) were added and incubated for 30 min on ice. Magnetic particles were isolated and the positive and negative cell fractions were resuspended in serum-free HBSS.

2.12. Cell proliferation

CD4$^+$ T cells (5.0×10^5) were cultured in the absence or presence of allogeneic CD45$^+$ or CD45$^-$ *E. tenella* Ag-binding cecal tonsil cells or macrophages pretreated with 25 μg/ml of mitomycin C (Sigma) at 1000:1, 200:1, 100:1, 50:1 and 25:1 stimulator:responder cell ratios. sIg$^+$ B cells were cultured with 10 ng/ml of LPS (Sigma) in the

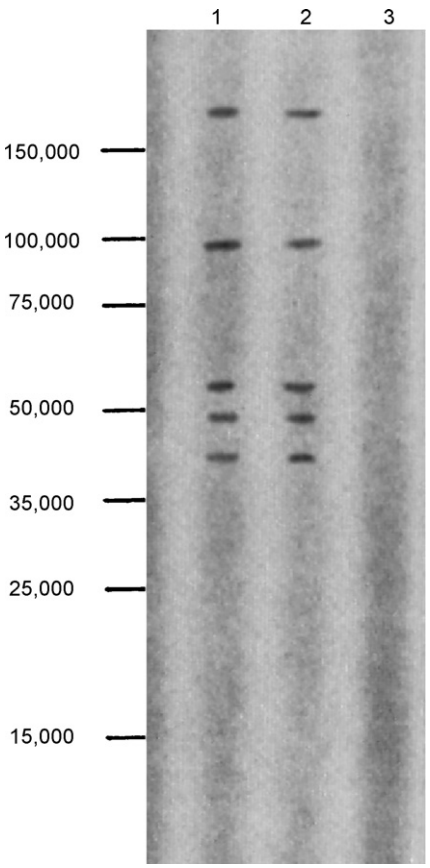

Fig. 1. Immunoblot analysis of isolated *E. tenella* sporozoites (lane 1), parasite Ag-binding cecal tonsils cells from infected chickens isolated by FACS using the *E. tenella* antiserum (lane 2), and cecal tonsil cells from uninfected chickens (lane 3). The migration of prestained molecular weight marker proteins is indicated on the left.

absence or presence of mitomycin C-treated $CD45^+$ or $CD45^-$ cells at the same ratios. Proliferation assays were carried out in round-bottom microtiter wells containing 0.2 ml of RPMI 1640 using the Cell Proliferation BrdU ELISA kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Control wells contained medium with either responder or stimulator cells alone.

2.13. IFN-γ and IgG ELISPOT

For IFN-γ quantification, 5.0×10^5 $CD4^+$ T cells were cultured in the absence or presence of 5.0×10^3 autologous $CD45^+$ or $CD45^-$ *E. tenella* Ag-binding cecal tonsil cells for 3 days. For IgG quantification, 5.0×10^5 sIg^+ B cells were cultured with 10 ng/ml of LPS in the absence or presence of 5.0×10^3 $CD45^+$ or $CD45^-$ cells for 10 days. Microplates were coated with anti-chicken IFN-γ (Yun et al., 2000) or anti-chicken IgG (Sigma) Abs at 4 °C overnight, blocked with PBS containing 10 mg/ml BSA and 0.05% Tween 20 at room temperature for 2 h, and washed 3 times with PBS. ELISPOT assays were performed for 24 h, the plates were washed, incubated for 24 h with biotinylated anti-chicken IFN-γ or anti-chicken IgG detection Abs, 2 h with horseradish peroxidase-streptavidin (1:2,000, Dako), and developed for 10–30 min with 200 μl of 0.3 mg/ml 3-amino-9-ethylcarbazole (Pierce, Rockford, IL). Colored spots were observed using a stereomicroscope and enumerated using an image analyser (IM50, Leica, Wetzlar, Germany). Control wells contained medium with T cells, B cells, $CD45^+$ or $CD45^-$ cells alone.

2.14. Statistical analysis

Mean ± SD values ($N = 3$) were calculated and differences between treatment groups were assessed using the Mann Whitney test, and considered significant at $p < 0.05$.

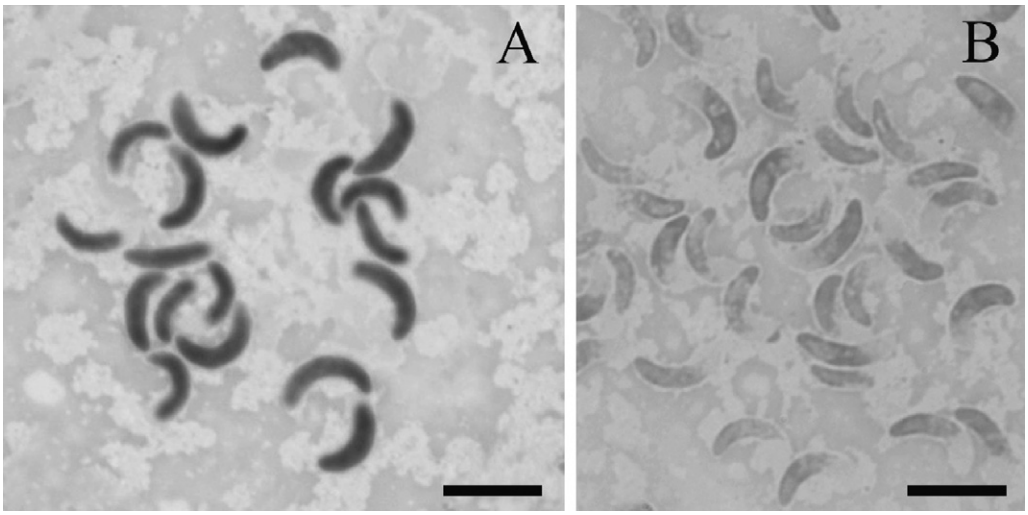

Fig. 2. Avidin–biotin peroxidase complex staining of ethanol-fixed sporozoites using *E. tenella*-immune antiserum (A) or a normal rabbit serum (B). Bars = 10 μm.

3. Results

3.1. Identification of E. tenella Ag-binding cells in cecal tonsils of parasite-infected chickens

Immunoblot analysis using an *E. tenella* antiserum revealed an identical pattern of five distinct bands with apparent molecular weights between 42,000 and 167,000 Da when comparing isolated sporozoites with cecal tonsil tissue from parasite-infected chickens (Fig. 1). The antigenic proteins were also detected by immunohistochemical staining of the sporozoites (Fig. 2). Immunofluorescence microscopy revealed that the parasite Ag-binding cells were localized in the lamina propria of the cecal tonsils within the germinal centers (Fig. 3A and B) and in the diffuse lymphoid tissue (Fig. 3A and B) where they were seen as groups of cells (Fig. 3A) and scattered beneath the epithelium lining the crypts of Lieberkuhm (Fig. 3B). Sporozoite Ag-binding cells also were detected in the epithelium between the intestinal epithelial cells (Fig. 3C).

By combined immunohistochemistry/transmission electron microscopy, cecal tonsil cells from *E. tenella*-infected chickens contained parasite Ags expressed exclusively on their surface (Fig. 4). Two types of cells

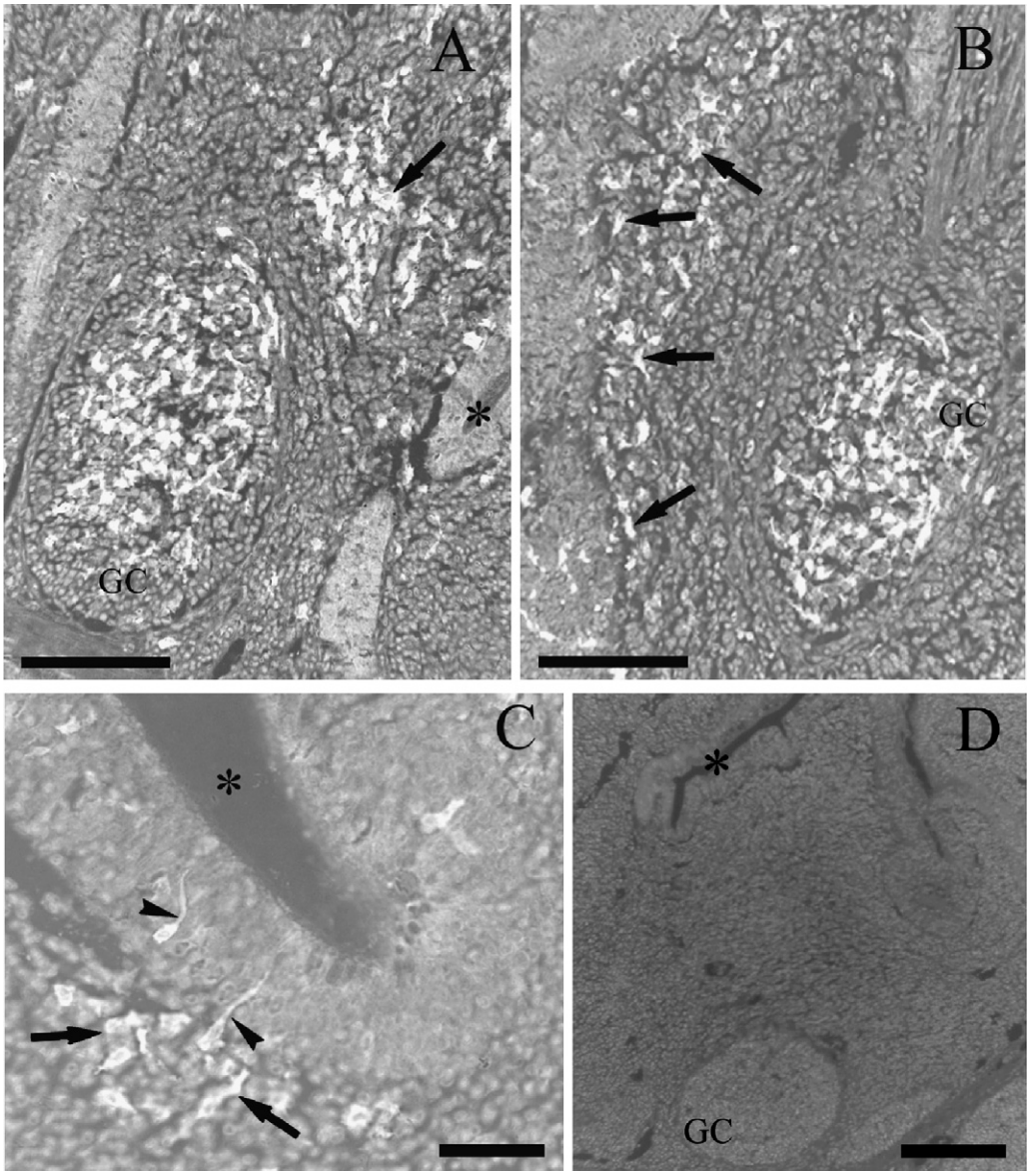

Fig. 3. Immunofluorescence images of *E. tenella* Ag-binding cells in the cecal tonsils of infected chickens. Tissue sections were stained with *E. tenella* sporozoite antiserum revealing parasite Ag-binding cells in the germinal centers (GC) (A and B), in the diffuse lymphoid tissues (A–C), and between epithelial cells lining the crypts of Lieberkhün (C). The arrows indicate sporozoite Ag-binding cells in the lamina propria and the arrowheads indicate sporozoite Ag-binding cells between epithelial cells. Note that the parasite Ag-binding cells in the diffuse lymphoid tissue appear as concentrated into groups (A) or scattered beneath the epithelium lining the crypts (arrows) (A–C). (D) Cecal tonsil from uninfected chicken stained with *E. tenella* antiserum. (*)lumen of the crypts. Bars in (A) and (B) = 70 μm; (C) = 25 μm; (D) = 60 μm.

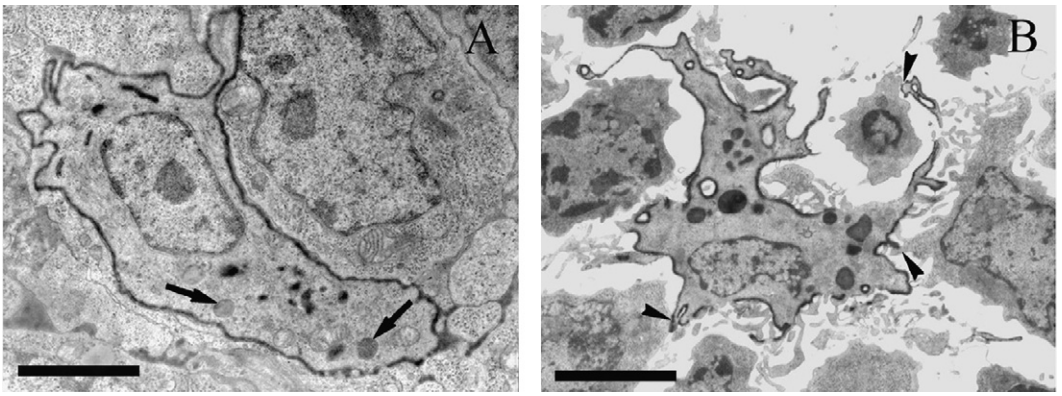

Fig. 4. Immunoelectron micrographs of *E. tenella* Ag-binding cells in the cecal tonsils of infected chickens. Tissue sections were reacted with *E. tenella* sporozoite antiserum and parasite-Ag-binding cells were visualized by avidin–biotin peroxidase complex staining. Note that the Ags recognized by the *E. tenella* antiserum appear on the cell surface. (A) Ag-binding cell located in the diffuse lymphoid tissue. The arrows indicate electron-dense cytoplasmic granules. Bar = 6 μm. (B) Ag-binding cells which are located within the germinal centres, exhibiting long and thin cytoplasmic processes in germinal centers. Note the intimate contact between the Ag-binding cell and neighbouring cells (arrowheads). Bar = 8 μm.

showing immunoreactivity on their surface were observed. The first was located in the diffuse lymphoid tissue and consisted of cells having an elongated shape with at least one process (Fig. 4A). The second, which was observed throughout the germinal centers, consisted of star-shaped cells with long and thin cytoplasmic processes that extended between neighbouring lymphoid cells (Fig. 4B). Short filiform processes were apparent, extending from both the cell body and the long cytoplasmic extensions that established close contacts with neighbouring lymphoid cells. Electron-dense granules occurred in the cytoplasm of both the elongate and the stellate cells. No parasite Ag immunoreactivity was found in the intercellular space, or associated with lymphocytes or macrophages (Fig. 5).

3.2. Isolation of *E. tenella* Ag-binding cecal tonsil cells

Fig. 6 shows representative immunofluorescence profiles of cecal tonsil cells stained with the *E. tenella* antiserum following collagenase digestion and Percoll density gradient isolation of the released cells from uninfected and infected chickens. A substantial fraction ($16.5 \pm 3.2\%$) of the cells from infected animals showed positive staining compared with 0% from the uninfected group. Logical gating based on the optical (forward/side scatter) and fluorescence profiles allowed isolation of the *E. tenella* Ag-binding cells (Fig. 7). By phase contrast microscopy, two morphologic cell types were distinguished, elongated cells with sparse cytoplasmic processes, and stellate cells with numerous thin cytoplasmic processes (Fig. 8). The sorted parasite Ag-binding cells were separated into $CD45^+$ and $CD45^-$ subpopulations and again examined morphologically. $CD45^+$ cells stained with an anti-CD45 Ab–biotin–streptavidin–peroxidase complex appeared elongated in shape with scarce cytoplasmic processes (Fig. 9A). Direct phase contrast examination of $CD45^-$ cells revealed a dendritic morphology, having large, thin, and slightly

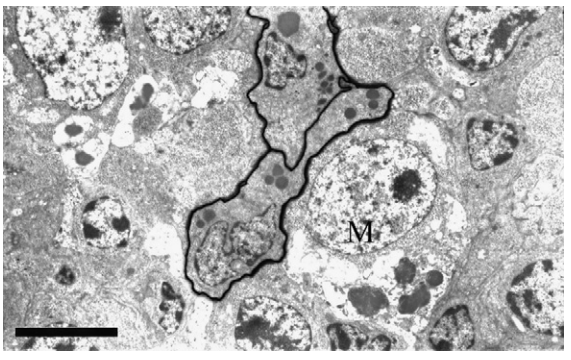

Fig. 5. Immunoelectron micrograph of the diffuse lymphoid tissue in the cecal tonsils of *E. tenella*-infected chickens. Note the positive immunoperoxidase reaction for the parasite Ag on the surface of the Ag-binding cell, whereas the lymphoid cells and macrophages (M) are devoid of peroxidase activity. Bar = 10 μm.

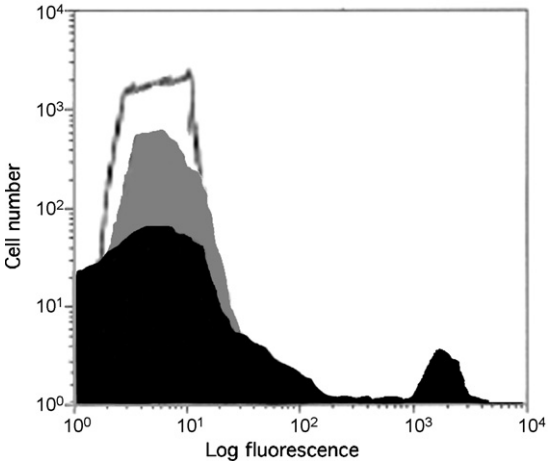

Fig. 6. Immunofluorescence histogram of the *Eimeria* antigen expression on cecal tonsil cells from *E. tenella*-infected chickens. The solid black and gray lines represent cells from infected and uninfected chickens, respectively. The broken line represents cells from infected chickens treated with secondary Ab alone.

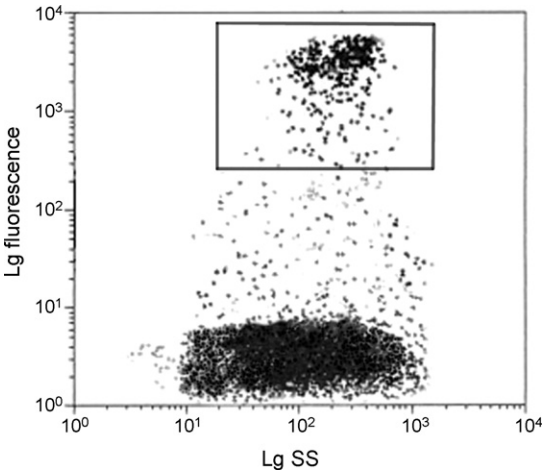

Fig. 7. Dot plot showing the scatter pattern of cecal tonsil cells from parasite-infected chickens stained with *E. tenella* antiserum and analyzed by FACS. The box indicates the sort region which was used for enriching *E. tenella* Ag-binding cecal tonsil cells.

branched cytoplasmic processes that extended in many directions from the cell body (Fig. 9B). Using this double Ab cell isolation protocol, we obtained 8.8×10^5 $CD45^+$ cells and 6.3×10^5 $CD45^-$ cells per chicken with approximately 95.6% purity and an average viability of 93% for both cell types.

3.3. Phenotypic and functional characterization of *E. tenella* Ag-binding cecal tonsil cells

By FACS analysis, the isolated $CD45^+$ cells showed high surface expression MHC class I, MHC class II, and selectin but lacked T cell, B cell, and macrophage markers (Table 1). $CD45^-$ cells expressed IgM, IgG, VCAM-1, and ICAM-1 but were negative for leukocyte, MHC, T cell, B cell, and macrophage markers. Functional studies showed that $CD45^+$ cells stimulated the proliferation of allogeneic naïve $CD4^+$ cells, while $CD45^-$ cells were incapable of stimulating proliferation (Fig. 10). Similarly, $CD45^+$ cells, but not $CD45^-$ cells, induced IFN-γ synthesis by autologous naïve $CD4^+$ cells (Fig. 11). By contrast, $CD45^-$ cells stimulated LPS-induced B cell proliferation and increased LPS-induced IgG secretion, while $CD45^+$ cells were unable to mediate either effect (Figs. 12 and 13).

4. Discussion

In this study, we demonstrated a novel method to isolate Ag-binding cells based on the specific binding of a polyclonal Ab against *E. tenella* proteins to parasite Ags expressed on the surface of cecal tonsil cells of *E. tenella*-infected chickens. The Ag-binding cells were demonstrated by immunofluorescence and immunoelectron microscopy techniques. These techniques allowed the visualization of two distinct populations of Ag-binding cells, which we propose to constitute FDCs and IDCs based upon the retention of parasite Ag on their surface as well as the appearance of three typical morphologic features of DCs that are distinguishable from other cells of the avian immune system. First, avian FDCs exhibit a dendritic morphology (Olah and Glick, 1979; Del Cacho et al., 1993a; Gallego et al., 1995b; Kroese et al., 1982) whereas IDCs have elongated cell bodies with irregular cytoplasmic processes (Del Cacho et al., 1993b, 1995). Second, both cells lack phagolysosomes (Olah and Glick, 1979; Eikelenboom et al., 1983). Finally, both cells possess electron-dense cytoplasmic granules (Olah and Glick, 1979, 1982; Del Cacho et al., 1995). In addition to the morphologic features, IDCs and FDCs can be distinguished based upon their differential expression of CD45. CD45 is a transmembrane glycoprotein expressed on chicken leukocytes and IDCs, but not FDCs (Del Cacho et al., 2008; Jeurissen et al., 1988a; Igyártó et al., 2007). In concert with the results of this investigation, $CD45^+$ cells had the morphologic appearance of IDCs and expressed MHC class I, MHC class II, and selectin, but did not express monocyte/macrophage markers such as CD156 and KUL01 (Glick and Olah, 1984; Yamamoto et al., 1999; Mast et al., 1998). On the other hand, our $CD45^-$ cells showed the morphologic character-

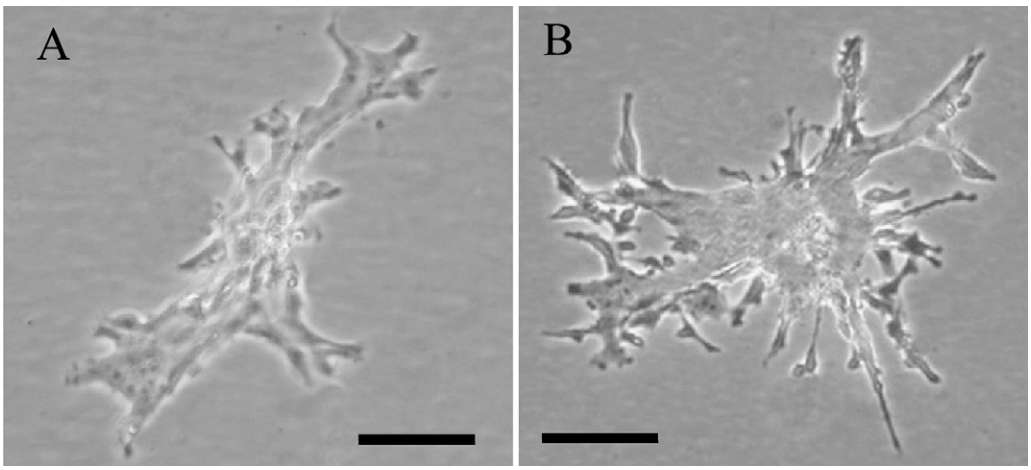

Fig. 8. Distinct morphological characterization of *E. tenella* Ag-binding cecal tonsil cells isolated by FACS from parasite-infected chickens visualized by phase contrast microscopy. (A) One cell type is characterized by an elongated shape with sparse and irregular cytoplasmic processes. (B) The second cell type is characterized by a stellate shape with numerous thin cytoplasmic processes. Bars = 7 μm.

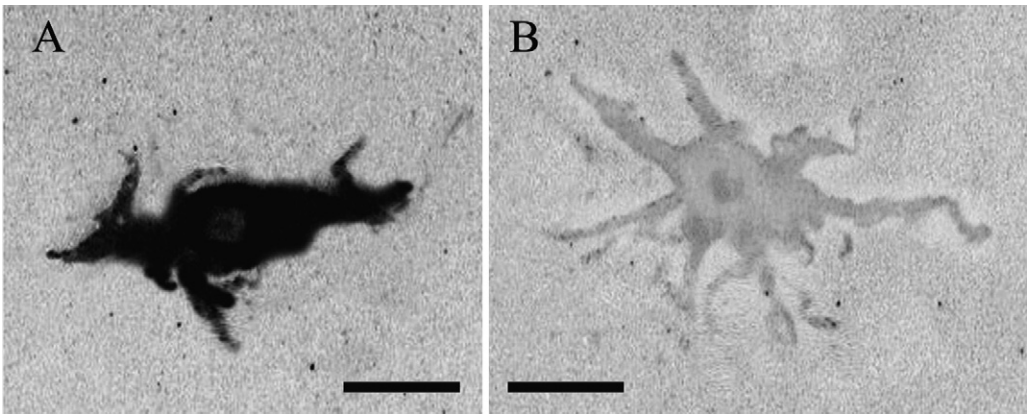

Fig. 9. Morphologies of *E. tenella* Ag-binding cecal tonsil cells separated according to CD45 expression. (A) $CD45^+$ cell showing an elongated shape and scarce processes. (B) $CD45^-$ cell displaying a dendritic morphology with large and thin cytoplasmic processes. Bars = 7 μm.

istics of FDCs and expressed surface IgG, IgM, ICAM-1, and VCAM-1, as reported by others (Briére et al., 2001; Banchereau and Steinman, 1998).

To determine whether or not $CD45^+$ and $CD45^-$ cells possessed the functional properties of mature IDCs and FDCs, we compared their abilities to stimulate T or B cell proliferation and to augment MLR-induced IFN-γ secretion or LPS-induced IgG secretion. Isolated $CD45^+$ cells induced the proliferation of naïve allogeneic $CD4^+$ cells and augmented the secretion of IFN-γ by allogeneic cells as expected of IDCs. Macrophages and FDCs are known to stimulate the proliferation of B cells and Ig synthesis (Fakher et al., 2001). Our finding that $CD45^-$ cells stimulated proliferation of and IgG production by allogeneic B cells in a MHC unrestricted manner supports the functional characteristic of FDCs. In mammals, the expression of MHC class II Ags on FDCs is debated (Fakher et al., 2001; Tew et al., 1990; Schriever and Nadler, 1992) although a recent study indicated that the expression of MHC class II Ags varies depending upon the maturation stage of FDCs (Muñóz-Fernández et al., 2006). In avians, all attempts to demonstrate the expression of MHC class II Ags on FDCs have, to date, failed (Del Cacho et al., 2008; Mast et al., 1998; Salomonsen et al., 1991).

MHC-restricted IFN-γ synthesis is characteristic of several types of APCs including IDCs, macrophages, and B cells (Dieli et al., 1997). IFN-γ, typically associated with Th1 type immune responses, is frequently used to test the capability of DCs to activate T cell-dependent responses because other Th1 cytokines (e.g. IL-2) are produced by a

Table 1
Phenotypic analysis of *E. tenella* Ag-binding cecal tonsil cells separated according to CD45 expression and macrophages.

Surface marker	mAb	$CD45^+$	$CD45^-$	Macrophages
Leukocyte marker				
CD44	AV6	++	–	+++
MHC molecules				
MHC class I	F21-2	+++	–	+++
MHC class II	2G11	+++	–	+++
T cell markers				
CD3	CT-3	–	–	–
CD4	CT-4	–	–	–
CD5	2-191	–	–	–
CD8	CT-8	–	–	–
B cell markers				
CD28	AV7	–	–	–
Bu-1	21-1A4	–	–	–
CD79	MH57	–	+	–
Immunoglobulins				
IgM	M-4	–	++	–
IgG	G-1	–	++	–
IgA	A-1	–	–	–
Monocyte/macrophage markers				
Anti-macrophage	KUL01	–	–	+++
CD156	CD156b	–	–	+++
Adhesion molecules				
CD41/CD61	11C3	+	+	+
CD51/CD61	LM609	+	+	+
VCAM-1	BBIG-V1	–	++	–
ICAM-1	BBA17	–	++	–
Selectin	+++	–	+	

Each + symbol represents approximately one $\log_{10}$ increased expression over background as determined by FACS analysis.

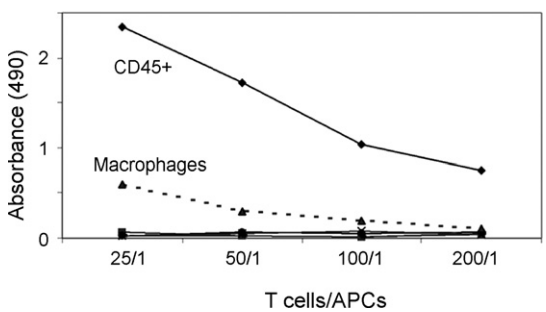

Fig. 10. Proliferative response of naïve $CD4^+$ T cells cultured with allogeneic $CD45^+$ or $CD45^-$ *E. tenella* Ag-binding cecal tonsil cells from parasite-infected chickens. The cells were mixed at various T cell/Ag-binding cell ratios and proliferation was measured by BrdU incorporation and quantified by absorbance at 490 nm. $CD4^+$ T cells plus $CD45^+$ cells (◆). $CD4^+$ T cells plus $CD45^-$ cells (■). $CD4^+$ T cells plus macrophages (▲). $CD4^+$ T cells alone (×). Each data point represents the mean of three replicates per sample with standard deviations <10% in all cases. Representative results from three experiments with similar results are shown.

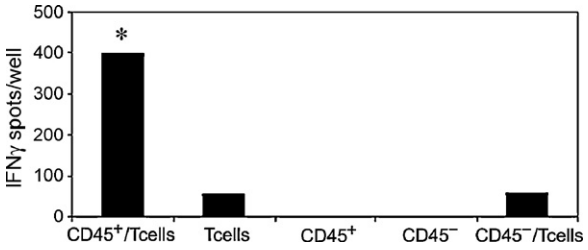

Fig. 11. IFN-γ secretion by naïve CD4$^+$ T cells cultured with autologous CD45$^+$ or CD45$^-$ *E. tenella* Ag-binding cecal tonsil cells from parasite-infected chickens. T cells alone or CD45$^+$ or CD45$^-$ cells alone, or T cells plus CD45$^+$ or CD45$^-$ cells were cultured for 3 days and the number of IFN-γ-secreting cells determined by ELISPOT assay. Each data bar represents the mean of three replicates per sample with standard deviations <10% in all cases. Representative results from three experiments with similar results are shown. (*) $p < 0.05$ when comparing CD4$^+$ T cells plus CD45$^+$ cells with CD4$^+$ T cells plus CD45$^-$ cells.

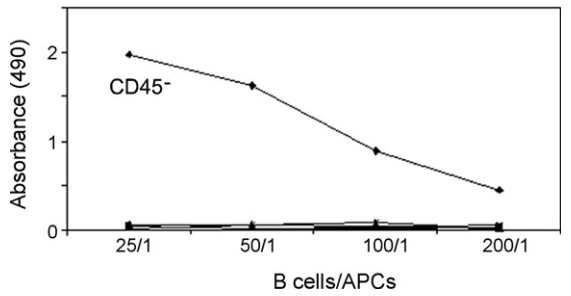

Fig. 12. LPS-stimulated proliferative response of B cells cultured with allogeneic CD45$^+$ or CD45$^-$ *E. tenella* Ag-binding cecal tonsil cells from parasite-infected chickens. The cells were mixed at various T cell/Ag-binding cell ratios and proliferation was measured by BrdU incorporation and quantified by absorbance at 490 nm. B cells plus CD45$^-$ cells (◆). B cells plus CD45$^+$ cells (■). B cells alone (×). Each data point represents the mean of three replicates per sample with standard deviations <10% in all cases. Representative results from three experiments with similar results are shown.

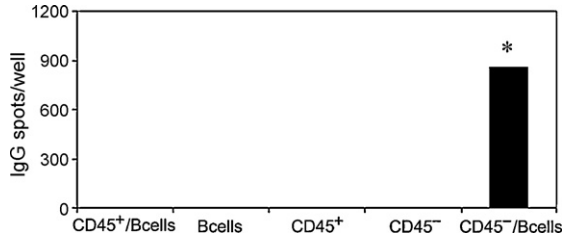

Fig. 13. LPS-stimulated IgG secretion by B cells cultured with autologous CD45$^+$ or CD45$^-$ *E. tenella* Ag-binding cecal tonsil cells from parasite-infected chickens. B cells alone or CD45$^+$ or CD45$^-$ cells alone, or B cells plus CD45$^+$ or CD45$^-$ cells were cultured for 10 days and the number of IgG-secreting cells determined by ELISPOT assay. Each data bar represents the mean of three replicates per sample with standard deviations <10% in all cases. Representative results from three experiments with similar results are shown. (*) $p < 0.05$ when comparing B cells plus CD45$^-$ cells with B cells plus CD45$^+$ cells.

much lower percentage of T cell subpopulation (Letsch and Scheibenbogen, 2003). In addition, CD45$^+$ cells stimulate allogeneic T cells in MLR. Although this is not a unique characteristic of IDCs, there is evidence that IDCs are the main stimulators of MLR (Fakher et al., 2001). The MLR results from the recognition of allogeneic MHC class II by the T cell receptor (Neefjes and Momburg, 1993) and IDCs are good stimulators due to their high level of MHC class II expression. Our results showed that isolated CD45$^+$ cells were more potent stimulators of MLR compared with CD45$^-$ cells and macrophages. Therefore, these results indicate that the isolated CD45$^+$ cells possess similar phenotypic and immunostimulatory properties as IDCs although these cells did not express specific markers of the mononuclear phagocyte system. In mammals, distinct DC subpopulations capable of T cell activation exist (Briére et al., 2001). Therefore, it is possible that a heterogeneous subpopulation of avian DCs capable of inducing T cell activation may include a DC subset which belongs to the mononuclear phagocyte system as described by Jeurissen et al. (1988b).

In summary, this is the first report that demonstrates the isolation of two distinct populations of *E. tenella* Ag-binding chicken cecal tonsils cells with morphologic, phenotypic, and functional characteristics of IDCs and FDCs. Further *in vitro* investigations are needed to precisely define the roles that these DCs play in the induction and regulation of host protective immunity in the gut.

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